AMOUNT, LOCATION, PRIMING CAPACITY, CIRCULARITY AND OTHER PROPERTIES OF CYTOPLASMIC DNA IN SEA URCHIN EGGS[1]

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The presence of large amounts of DNA in the cytoplasm of the mature egg of many species of animals has been reported by a number of early workers (for summaries see Brachet, 1962; Haggis, 1964; Grant, 1965; Monroy, 1965; Tyler and Tyler, 1966b). Values several hundred times that of the nucleus have been reported in eggs of sea urchins and frogs which have been the most extensively investigated material. However, the methods employed in the early determinations did not clearly distinguish between DNA and materials, such as polysaccharides and RNAs, that might interfere with the determinations, and, in fact, as the methods have become more refined the reported values have dropped. Thus, in *Paracentrotus lividus* Hoff-Jørgensen (1954) obtained about 20 times the haploid (H) value by microbiological assay and Whiteley and Baltzer (1958) obtained values at the 32-cell stage by a fluorometric method that extrapolate to a similar value for the unfertilized egg. In *Hemicentrotus lividus* Sugino *et al.* (1960) reported about 37 × H on the basis of thymidine determinations. Pikó and Tyler (1965) obtained approximately 13 × H and 8 × H, respectively, in *Lytechinus pictus* and *Strongylocentrotus purpuratus* by differential and buoyant density centrifugation methods. Eberhard and Mazia (1965), from fluorometric measurements, estimated about 180 × H in *S. purpuratus* but indicated that the material that reacted with the 3,5-diaminobenzoic acid dihydrochloride in their experiments might not all be DNA. Baltus *et al.* (1965), using a microfluorometric method, and Bibring *et al.* (1965), using centrifugation methods, found about 25 × H in *Arbacia lixula*.

The location of the egg cytoplasmic DNA has also been uncertain. A few years ago substantial evidence first appeared for the presence of DNA in the mitochondria of cells of a number of organisms, including chick embryo (Chèvremont, 1962; Nass and Nass, 1963), mammalian tissues (Swift *et al.*, 1964; Schatz *et al.*, 1964b), protozoa (Steinert *et al.*, 1958; Rudzinska *et al.*, 1964), molds (Luck and Reich, 1964), yeast (Schatz *et al.*, 1964a), ferns (Bell and Mühlethaler, 1964), maize (Ris, 1962). The evidence has accumulated since these first investigations and DNA is now generally considered to be an integral part of the mitochondrion (for reviews and further evidence see Gibor and Granick, 1964; Swift, 1965; Nass

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et al., 1965; Rabinowitz *et al.*, 1965; Suyama and Preer, 1965; Dawid, 1966; Corneo *et al.*, 1966; Sinclair and Stevens, 1966). It seemed possible, then, that the egg cytoplasmic DNA might be entirely contained in these bodies. In fact a calculation (Pikó and Tyler, 1965) based upon an estimate of the volume occupied by mitochondria (*ca.* 9%) in sea urchin eggs (Shaver, 1956, 1957) and reported values (*e.g.*, Schatz *et al.*, 1964a) of DNA in mitochondria, gives approximately the amount of cytoplasmic DNA that has been found.

Baltus and Brachet (1962) (*cf.* Roller, 1963; Brachet, 1965) found about two-thirds, at least, of the cytoplasmic DNA of frog's eggs to be associated with large particles (pigment granules and yolk platelets) that sediment at low speed. Yolk spherules of certain types, however, have been shown to be derived from mitochondria at least in some species (Lanzavecchia, 1960, 1965; Ward, 1962; *cf.* Srivastava, 1965). In eggs of the clawed toad *Xenopus laevis*, and the frog *Rana pipiens*, Dawid (1965, 1966) has found DNA in the mitochondria. The total obtained in the mitochondrial preparations accounts for some 65 to 80% of the cytoplasmic DNA.

Comparisons have been made of cytoplasmic with nuclear DNA with respect particularly to buoyant density and estimated molecular weight. Bibring *et al.* (1965) indicate that in *P. lividus* it has a high molecular weight and a base composition similar to that of nuclear DNA. They also report that the buoyant density of the DNA (presumably mostly cytoplasmic) extracted from eggs is similar to that found in sperm. Carden *et al.* (1965) also found in *Arbacia punctulata* that nuclear and cytoplasmic DNAs exhibit the same buoyant density in CsCl gradients. In the experiments reported here the cytoplasmic DNA is found to differ in buoyant density from that of the nucleus.

The sedimentation behavior of this material indicated a similarity to the DNAs of various viruses that are known to have a closed circular structure of uniform circumference, as shown electron microscopically by Weil and Vinograd (1963) for the DNA of the polyoma virus and by Kleinschmidt *et al.* (1963) and Chandler *et al.* (1964) for the replicating form of bacteriophage ØX174. Also, as Vinograd *et al.* (1965) have shown, the circles are composed of double helices that are in a superhelical form unless scissions are introduced into one or the other of the two strands. Circular DNAs with circumferences ranging from 0.5 to 9.7 microns have been found by electron microscopy in DNA preparations from boar sperm by Hotta and Bassel (1965). Recent studies by Borst and Ruttenberg (1966) and by Van Bruggen *et al.* (1966) have shown the presence of circular DNA in mitochondrial preparations from chick and mouse liver and from beef heart. The circles were of uniform circumference (*ca.* 5.45 microns). Sedimentation velocity analyses revealed two components with standard sedimentation coefficients of 39–42S and 27–29S that correspond to the twisted and relaxed circular forms described by Vinograd *et al.* (1965). Similar findings have been reported by Sinclair and Stevens (1966) for mouse liver mitochondria. Our own studies indicate that the DNA of the mitochondria of sea urchin eggs is also of the circular type as will be reported here and in more detail in a subsequent paper.

Evidence that cytoplasmic DNA may be potentially active in oocytes or mature eggs has been provided by experiments of Shmerling (1965) on sturgeon oocytes, showing that DNA extracts that must contain predominantly cytoplasmic DNA

possessed priming activity for DNA and for RNA synthesis equal to that of DNA extracted from the sperm. There is also evidence for *in vivo* activity of the cytoplasmic DNA of eggs. Thus Mezger-Freed (1963) has reported that artificially activated enucleated frog eggs (with the nucleus in an attached exovate) synthesize DNA about as rapidly as do the fertilized eggs during early cleavage. Similarly in sea urchins Baltus *et al.* (1965) report synthesis of DNA by artificially activated non-nucleate fragments and, in addition, the synthesis of RNA. In the present experiments the DNA extracted from the mitochondria of sea urchin eggs was found to be capable of serving as primer for RNA synthesis.

Materials and Methods[4]

(A) *Egg and sperm samples*

The sea urchins *Lytechinus pictus* and *Strongylocentrotus purpuratus* were used in these experiments. The eggs were obtained by KCl injection and handled in artificial sea water as described elsewhere (Tyler and Tyler, 1966a). After removal of the gelatinous coat in pH 5 sea water and thorough washing the eggs were suspended in 0.55 *M* KCl following three approximately 30:1 (v/v) washings in this solution. Samples were removed from the penultimate suspension for counting (Tyler and Tyler, 1966a).

"Dry" sperm (semen) was collected as it exuded freely from dissected gonads and diluted with artificial sea water to a stock solution of 0.5 to 1.0%. Spermatozoal counts were made with the Coulter electronic counter having a 30 μ orifice. As a check, counts were also made by hemocytometer.

(B) *Preparation of homogenates*

The preparative procedure was similar in principle to that of Kay (1964); namely, the use of detergent followed by salt extraction. In addition EDTA was included further to reduce the possibility of nuclease activity and to serve as a buffer supplemental to the egg material itself. In these experiments one volume of packed KCl-washed eggs was mixed with three volumes of the homogenization medium [4% sodium dodecyl sulphate (SDS), 0.08 *M* ethylenediamine tetraacetate and 9% ethanol, pH 7.8] and stirred gently for 20 to 30 minutes at 20° C., with a Teflon rod. CsCl (optical grade, Harshaw Chemical Company) was then added, with continued slow stirring for about one-half hour, to give the desired final densities. These were 1.5 gm./cm.^{3} in the initial experiments in which attempts were made by differential centrifugation to remove Dische-interfering materials and 1.70 gm./cm.^{3} in the later experiments in which the DNA was isolated by buoyant density centrifugation. Marker C^{14}-DNA (see below) dissolved in 3.75 molal CsCl, when used, was added at this time in the ratio of 1 volume to 150 or 300 volumes of homogenate. In some cases the homogenates were stored at

[4] *Abbreviations used in the text:* DNA, deoxyribonucleic acid; RNA, ribonucleic acid; DNase, pancreatic deoxyribonuclease; SDS, sodium dodecyl sulphate; EDTA, ethylenediamine tetraacetate; TCA, trichloroacetic acid; SSC, standard saline-citrate (0.15 *M* NaCl, 0.015 *M* Na citrate, pH 7); ATP, GTP, UTP, CTP, adenosine-, guanosine-, uridine-, and cytidine-triphosphate, respectively.

— 20° C. before the addition of CsCl, and this had no apparent effect on the amount of DNA later obtained or on its properties.

(C) *Marker DNA*

Fertilized *Lytechinus pictus* eggs, at a density of 40,000 eggs/ml., were raised until the gastrula stage in artificial sea water containing 50 μC./ml. C^{14}-thymidine (30 μC./μM specific activity, New England Nuclear Corp.). After several washings with ice-cold sea water and 0.55 *M* KCl, the embryos were homogenized with SDS-EDTA and the DNA was isolated by buoyant density centrifugation in CsCl solution. Fractions of 0.15 ml. were collected by puncturing near the bottom of the centrifuge tubes and examined for absorption at 260 mμ and for radioactivity. A single sharp peak of absorption, and of radioactivity, was obtained. The fractions containing this material were pooled and stored in a freezer at — 20° C. In two separate preparations made in this manner one had a specific activity of 12×10^{-6} mC./mg. DNA and the other 16×10^{-6}. In the experiments involving the use of marker DNA the amount of radioactivity was measured in a Packard Tri-Carb scintillation counter at 50% counting efficiency by a method described elsewhere (Tyler, 1966).

(D) *DNA determinations*

Measurements of the DNA content of the various preparations in the initial experiments were made by the diphenylamine reaction of Dische (1930). This was used on hot acid extracts (15 minutes extraction with an equal volume of 10% TCA in a boiling water bath) of spermatozoal suspensions, and egg homogenates and the various fractions thereof. Aliquots, mixed with Dische's reagent and blank, respectively, were heated in a boiling water bath for 10 minutes, cooled rapidly and allowed to stand at room temperature for one hour. The absorptions were read with a Beckman DU spectrophotometer and DNA values calculated by comparison with a deoxyadenosine standard. In some cases, spectral absorption curves between 500 and 700 mμ were taken with a Cary Model 15 spectrophotometer.

For the purified materials isolated by the centrifugation methods the DNA values were determined directly from measurements of the absorption at 260 mμ, assuming a value (Ogur and Rosen, 1950) of 0.050 mg./O.D. unit.

(E) *Centrifugation*

All high speed centrifugations were done in a Spinco model L or L2-65 ultracentrifuge with an SW-39 or SW-65 rotor. The conditions of centrifugation are indicated in the individual experiments. The preformed density gradients employed in these experiments were prepared by use of a gradient-making device of the type described by Leif and Vinograd (1964) but capable of filling three tubes at one time.

The results of the buoyant density experiments were evaluated by the procedures described by Vinograd and Hearst (1962). All buoyant densities were calculated by the marker method. A value of 1.710 gm./cm.^{3} for *E. coli* DNA was assumed.

(F) *Isolation of mitochondria*

Following the experience of Shaver (1956) with the sea urchins used in these experiments, mitochondrial preparations were made by homogenization of KCl-washed eggs in 3 volumes of a solution containing 0.3 *M* sucrose, 0.36 *M* KCl, 0.03 *M* Tris-HCl and 0.003 *M* EDTA at pH 7.6. Homogenization was done by hand with a loose fitting Teflon pestle in a Potter-Elvehjem tube of about 2 cm. diameter containing 15 ml. of suspension, for a total of 10 strokes in the cold at relatively slow speed. The homogenates were first centrifuged for 10 minutes at 1200 to 1500 rpm (SW-25 rotor of the Spinco model L centrifuge, 4° C.) to remove nuclei and large egg fragments. The supernatant was then centrifuged at 12,000 rpm in the same rotor for 20 minutes. The resulting pellet was resuspended in homogenization medium and recentrifuged under the same conditions. Further processing is described below.

(G) *Priming activity*

The ability of the extracted DNA to serve as primer for RNA synthesis as first described by Weiss and Gladstone (1959), was measured from the incorporation of C^{14}-labeled CTP in a system containing also the other three trinucleotides (GTP, ATP, UTP), and an RNA polymerase prepared from *E. coli* by the method of Chamberlain and Berg (1962). We are indebted to Dr. K. Marushige for these materials.

Results and Comments

(A) *DNA content*

(1) *Determination by Dische reaction*

When the "DNA" of homogenates of whole eggs of *S. purpuratus* and *L. pictus* was directly assayed by the Dische reaction, the amount per cell ranged from 90 to 150 times that of the sperm. These are similar to the values reported by a number of earlier investigators as noted above. However, as Elson *et al.* (1954) noted with sea urchin eggs, the color that is obtained is not that characteristic of deoxyribose alone. In our tests the reaction mixture showed an absorption peak at 530 mμ. Figure 1 illustrates an absorption curve for the egg material (curve A) along with that of the sperm (D) and one for deoxyadenosine (F).

One initial attempt to remove the interfering material was done by differential centrifugation of the homogenates brought to a density of 1.5 gm./cm.^{3} with CsCl. After 14 hours of centrifugation at 35,000 rpm (Spinco SW-39 rotor) the material separated into a small gelatinous bottom pellet, a rubbery top layer (occupying about 5% of the tube when the final homogenate contains some 12 to 14% of eggs) and a clear intermediate fluid. Dische reactions were run on the combined pellet and intermediate fluid and on the top layer in the two species. Determinations were also made on sperm. The top layer contained about four-fifths of the 595 mμ absorption values of whole egg homogenates but the absorption curve is similarly abnormal (curve B of Figure 1). The possibility that "trapped" DNA may be contained in it is considered in a later section. For the combined pellet and clear

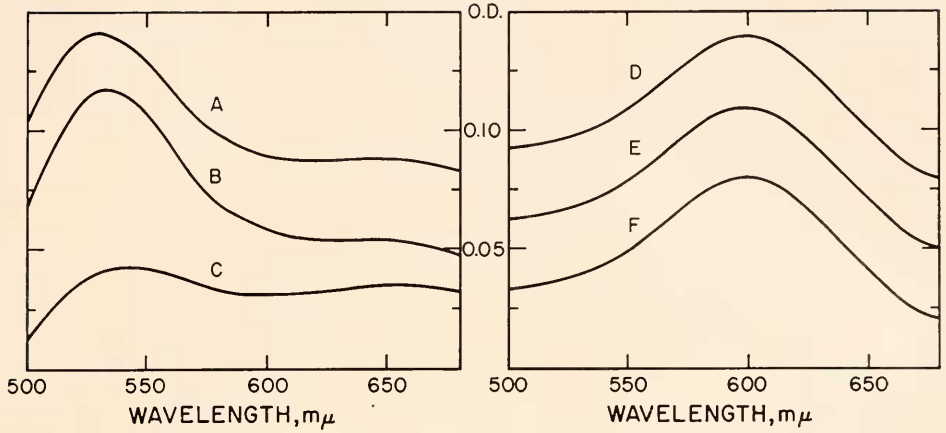

FIGURE 1. Spectral absorption curves of colors obtained with the Dische reaction. (A) Whole egg homogenate of *S. purpuratus;* (B) top layer and (C) combined pellet and clear layer obtained after differential centrifugation of homogenates of *S. purpuratus* eggs (see text); (D) sperm of *L. pictus;* (E) pre-purified and alpha amylase-treated extract from eggs of *L. pictus;* (F) deoxyadenosine standard.

layer the following values (in micrograms "DNA" per million eggs) were obtained:

S. purpuratus	17.7, 19.3, 20.5, 22.0; av. 19.9
L. pictus	19.6, 16.2, 16.4; av. 17.4

For the sperm the values were as follows:

S. purpuratus	0.72, 0.82; av. 0.77
L. pictus	0.84, 0.90; av. 0.87

The spectrum of the Dische-reacted combined pellet and clear layer (curve C of Figure 1) is somewhat less abnormal than that of the other preparations.

After exploration of a number of methods of disposing of the interfering material the use of alpha amylase proved effective. Preparations that are obtained by a single buoyant density separation show a turbid polysaccharide layer (see section 3) at the same level of the tube where the DNA is located. When this fraction, after it is precipitated with alcohol and redissolved, is treated with alpha amylase, as described in section 3, it gives a typical deoxyribose spectrum in the Dische reaction, as illustrated in curve E of Figure 1. By this procedure the DNA values obtained for the two species were:

L. pictus	8.0 pg./egg
S. purpuratus	3.6 pg./egg

These are similar to the values obtained by UV absorption measurements on purified DNA as described below.

(2) *Evidence against "trapping" of DNA in the top layer*

In order to examine the possibility that DNA might be trapped in the top layer that forms upon centrifugation of homogenates in the CsCl solutions, use was

made of the C^{14}-labeled gastrula DNA. In five experiments with *L. pictus* a sample of the labeled DNA was added to the homogenate and determinations made of the distribution between the top layer and the clear fluid after prolonged centrifugation in the CsCl solutions employed for buoyant density separations (see below). The values obtained in these experiments for the ratio of labeled DNA in the top layer to that in the clear fluid ranged from 1:99 to 2:98.

(3) *Direct determinations on purified material*

Preparations of DNA were made from whole eggs of *S. purpuratus* (3 experiments) and *L. pictus* (5 experiments) by buoyant density centrifugation of homogenates adjusted to a density of 1.70 gm./cm.^{3} with CsCl and containing radioactive (marker) gastrula DNA. Centrifugation was for at least 50 hours at 35,000 rpm at 10–12° C. in the SW-39 rotor of the Spinco model L centrifuge. A small amount of solid CsCl is present at the bottom of the tube at the end of the run. For collecting the fractions, then, the hypodermic needle is introduced above this layer (approximately 7 mm. from the bottom). Usually 15 to 20 fractions were collected and the radioactivity of small aliquots determined. The fractions comprising and surrounding those with the radioactive DNA were pooled and CsCl solution (1.70 gm./cm.^{3}; in 0.02 *M* Tris–HCl pH 7.6) added so as to give a volume sufficient to fill the centrifuge tubes, which were then re-run as before.

It was noted, early in these experiments, that a band of visible turbidity, later identified as polysaccharide (Pikó and Tyler, 1965; Segovia *et al.*, 1965), appeared in the region of the centrifuge tube where the marker DNA was located. This material continued to appear at the level of the DNA upon repeated centrifugations and it obscured the $O.D._{260}$ readings. For removal of this material two methods were explored. One was centrifugation of alcohol-precipitated and redissolved fractions on preformed CsCl density gradients (1.22 to 1.65 gm./cm.^{3} for 4 hours at 35,000 rpm, 20° C.) in which the polysaccharide sediments (*ca.* 100S) well ahead of most of the DNA. The other method was simply to incubate a solution (0.5 *M* KCl, 0.01 *M* Tris, 0.005 *M* EDTA, pH 7) of the alcohol-precipitated DNA and polysaccharide-containing fractions with α-amylase (Worthington, 2 × crystallized, at 0.75 mg./ml. for 1 hour at 37° C.). This method proved to be the more effective. The digestion with alpha amylase was generally done with the fractions collected after the first or second centrifugation. Following this the buoyant density centrifugations and collection of the fractions were repeated two times.

After the final buoyant density centrifugation, absorbances of the fractions at 260 and 280 mμ were read on a Beckman DU spectrophotometer. Radioactivity (of the marker DNA) determinations on aliquots again served to locate the DNA-containing region, and to provide an additional basis for quantitation. The fractions collected after the final centrifugation by this procedure showed a single $O.D._{260}$ peak at a level corresponding to a density near 1.70 gm./cm.^{3} The marker DNA showed a single peak of radioactivity in the region of 1.69. This is illustrated in Figures 2a and 2b. In one of these experiments an aliquot was treated with DNase (Worthington, electrophoretically purified, 0.1 mg./ ml. at 37° C. for 30 minutes) before the final buoyant density centrifugation. This resulted in complete elimination of both the $O.D._{260}$, and the radioactivity, peaks.

DNA prepared in this way shows a typical absorption spectrum, as illustrated in Figure 3. In 5 experiments with unfertilized eggs of *L. pictus* and 3 experiments with *S. purpuratus*, in which the determinations were made by this method, the following values were obtained for the content of DNA in micrograms per million eggs.

L. pictus	7.9, 9.3, 7.5, 8.2, 8.4; av. 8.26 ± 0.30
S. purpuratus	3.5, 2.8, 3.6; av. 3.30 ± 0.25

(4) *Extraction of DNA from eggs labeled during oogenesis*

Further evidence for the effectiveness of the extraction procedure has been obtained in an experiment in which the DNA was labeled radioactively during oogenesis by the general procedure described by Tyler and Tyler (1966a). In

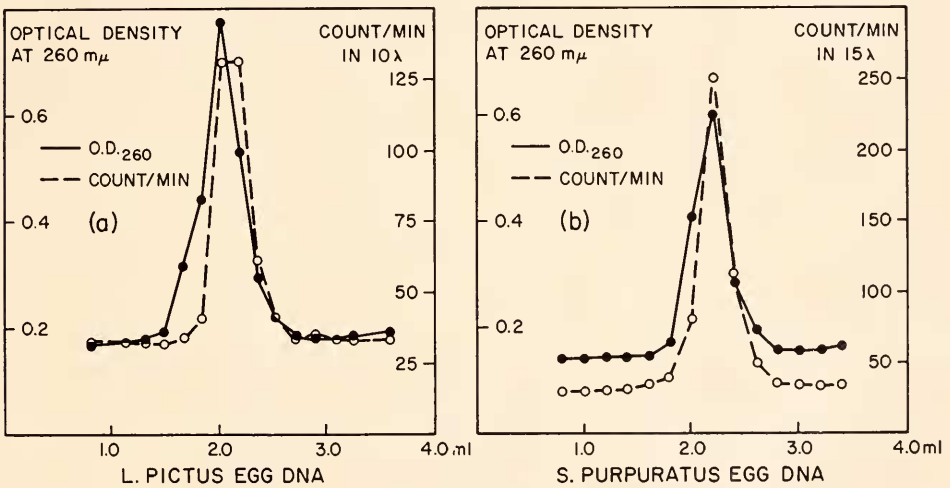

FIGURE 2. Cesium chloride gradients of DNA from unfertilized eggs of *L. pictus* and *S. purpuratus*. The C^{14}-labeled nuclear marker DNA (from *L. pictus* gastrulae) bands at a somewhat lower density than the bulk of the cytoplasmic DNA (for procedures see text).

this experiment a female *L. pictus* received two successive injections, intracoelomically, of 200 microcuries of H^3-thymidine (6 curies/mM) at a one-month interval and the eggs were collected one month after the second injection. About 100,000 eggs were obtained, and tests on an aliquot showed about two-thirds of the radioactivity to be in acid-precipitable form. Upon extraction by the procedures employed here (see section 3) all the labeled material, that was identified as DNA, was found, upon buoyant density centrifugation, to be in a layer at a density near 1.70 gm./cm.[3] This material contained about 0.1% of the originally injected radioactivity. In addition there was an approximately equal amount of radioactivity at the top of the tube. This material, upon treatment with preincubated (1 hour) pronase (Calbiochem, final concentration 2 mg./ml. in 0.25 *M* CsCl, 0.005 *M* Tris, 0.001 *M* EDTA, 5% ethanol, pH 7.6; incubated at 50° C. for 12 hours) lost more than 90% of its originally acid-precipitable radioactivity. It

may, then, be concluded that the extraction procedure yields practically all the DNA obtainable from the egg.

(B) *Presence of DNA in mitochondria and yolk*

Two sets of experiments were run in which homogenates of *L. pictus* eggs (of determined number) were subjected to differential centrifugation, as described under Methods, so as to separate a $250 \times g$ nuclear (N) pellet, a $18{,}000 \times g$ mitochondria + yolk (M + Y) pellet and a supernatant (S) fraction. Two or three consecutive buoyant density centrifugations in CsCl solution were performed

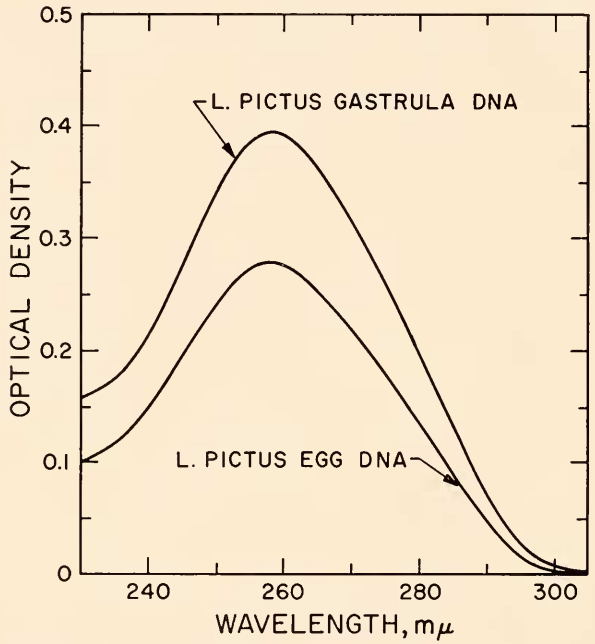

FIGURE 3. Ultraviolet absorption spectra of purified DNA in 0.015 *M* NaCl–0.0015 *M* sodium citrate, pH 7, from gastrulae and unfertilized eggs of *L. pictus*.

on each fraction, as described in section A3, the treatment with alpha amylase being applied on the fractions collected after the first centrifugation. From $O.D._{260}$ readings the following amounts of DNA in micrograms per million eggs were obtained. The distribution in per cent of the total is given in parentheses.

Experiment 1: N = 1.51 (20.5%); M + Y = 5.03 (68%); S = 0.85 (11.5%)
Experiment 2: N = 0.51 (8.5%); M + Y = 3.84 (65%); S = 1.57 (26.5%)

In experiment 1 no separate determination of DNA content of whole eggs was made, but if the average value of 8.26 pg. per egg from other experiments is taken then the recovery here is about 90%. In experiment 2 parallel determination of DNA content was made on an aliquot of the initial whole egg homogenate. This

gave a value of 8.40 micrograms per 10^6 eggs, as corrected for 76% recovery of marker DNA. If we assume a similar recovery for the above fractions then the corrected total for them is 7.8 micrograms, which would indicate very little, if any, loss in the fractionation procedure.

As the results show, the bulk of the DNA is found in the M + Y fraction. The DNA content of the N fraction is lower in experiment 2 than in experiment 1. In experiment 1 this fraction had not been washed. Microscopic examination has shown that some mitochondria and yolk spherules do sediment with this fraction. This probably accounts, then, for the value of its DNA content being almost twice that expected for the nuclei alone.

The S fraction is free of microscopically visible mitochondria and yolk particles, and shows in the two experiments 11.5 and 26.5%, respectively, of the total DNA content extracted from the eggs. Whether or not this DNA may be derived from damaged mitochondria or yolk cannot be stated at present.

An aliquot of the M + Y fraction in the second experiment, with *L. pictus*, was subjected to further processing by centrifugation on preformed linear gradients of sucrose solutions (from 0.93 *M* to 1.88 *M* sucrose in 0.003 *M* Tris, 0.0025 *M* EDTA, pH 7.6) in the SW-25 rotor of the Spinco model L at 25,000 rpm for 2 hours at 4° C., 2.5 ml. of the suspension of M + Y fraction being layered on 25 ml. of gradient in each tube. Under these conditions the yolk (Y) remains on the top of the gradient while the mitochondria (M) sediment as a band that is visible as a cloudy layer some 4 mm. wide at a region of the tube where, as determined by subsequent weighing, the density is 1.18 gm./cm.^{3} This fraction and the top one were collected, diluted with three volumes of 0.5 *M* KCl (containing 0.05 *M* Tris, 0.005 *M* EDTA, pH 7.6) and centrifuged at 12,000 rpm (SW-25 rotor) for 20 minutes. The pellets were suspended in SDS-EDTA solution, CsCl added to a density of 1.70 gm./cm.^{3} and the solutions subjected to two buoyant density centrifugations and fractionations, with intervening alpha amylase digestion, as described previously. The following values were obtained for DNA in micrograms per million eggs.

Experiment 2: M = 2.07; Y = 0.72

The sum represents 73% of the amount of DNA present in the M + Y fraction, as listed above.

In a separate experiment (3) an M + Y fraction of *L. pictus* eggs was prepared and all of this used for preparation of M and Y fractions as described in this section. The following values for DNA content (micrograms per million eggs originally extracted) were obtained.

Experiment 3: M = 2.47; Y = 0.35

From these experiments it is clear that the bulk of the DNA appears in the mitochondrial fraction. In the two experiments (2 and 3) in which M + Y was separated into M and Y the ratios (M:Y) of DNA content were 3:1 and 7:1, respectively. From the sedimentation behavior, including the wide separation of the two fractions, and from microscopic examination it is unlikely that the yolk fraction contains any significant amount of mitochondria as such. Considering also the lack of any appreciable trapping of marker DNA in that layer it is most reason-

able to conclude that the DNA found therein is a component of the yolk spherules. The differences in the relative amounts of DNA obtained from the mitochondrial and yolk fractions in the two experiments may be explained by the sensitivity of these particles to damage during the extraction procedures. The results of cesium chloride buoyant density centrifugation of DNA from mitochondria and from yolk are illustrated in Figure 4a, 4b. Both DNAs behave similarly, forming bands at somewhat higher density than the added radioactively labeled nuclear DNA.

In a preparation made by Dr. E. R. Berger, now of the Veterans Administration Hospital, Sepulveda, approximately 2140 mitochondria and 2280 yolk spherules were counted on a montage of electron micrographs of a thin section (maximum diameter) of an egg of *L. pictus*. From these figures, and values of 2.0 microns for the diameter of a yolk spherule and equivalent spherical diameter of 0.8 micron

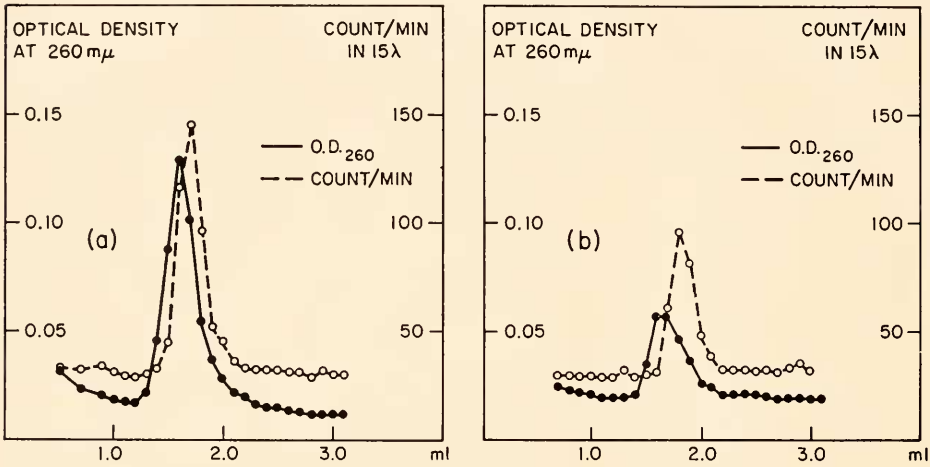

FIGURE 4. Cesium chloride gradients of DNA from (a) isolated mitochondria and (b) yolk of *L. pictus*. Each preparation contained added C^{14}-labeled marker DNA from *L. pictus* gastrulae. The band of radioactivity is at a lower density than that of $O.D._{260}$ absorption for both the mitochondrial and yolk-DNA.

for a mitochondrion, it may be estimated that there are some 80,000 yolk spherules and some 200,000 mitochondria per egg. This corresponds to the approximately 3:1 ratio for the DNA found in mitochondria and yolk in experiment 2.

(C) *Priming activity for RNA synthesis*

Purified DNA from eggs of *L. pictus* and *S. purpuratus* was tested for ability to serve as primer for RNA synthesis. DNA that is preponderantly (more than 99%) nuclear was prepared from late blastulae and from plutei of *S. purpuratus*, to serve as a basis for comparison. The measurements were made of the incorporation of radioactive label into RNA (material precipitable by 10% trichloroacetic acid) in a system containing, in 0.25 ml., the following: 0.1 μmole C^{14}-cytosine triphosphate (1.4 $\mu C./\mu M$), 10 μmoles Tris buffer, pH 8, 1 μmole $MgCl_2$, 0.25 μmole $MnCl_2$, 3 μmoles beta mercaptoethanol, 0.1 μmole each of ATP, GTP and

UTP, and the RNA polymerase from *E. coli*. The following values were obtained in terms of counts per minute (cpm at 30% counting efficiency) per 5 μg. DNA above a background of about 90 cpm for the complete mixture minus the DNA.

S. purpuratus:	Unfertilized egg DNA (80% cytoplasmic)	= 4,056 cpm
	Blastula DNA (< 1% cytoplasmic)	= 3,618 cpm
	Pluteus DNA (< 1% cytoplasmic)	= 4,305 cpm
L. pictus:	Unfertilized egg DNA (90% cytoplasmic)	= 3,027 cpm

These initial values are all in the same general range. If only the nuclear DNA of the egg preparations were active, the values for these preparations would have been very much lower (one-fifth to one-tenth of those obtained). It may be concluded, then, that the cytoplasmic DNA can function as primer for RNA synthesis.

(D) *Some physical properties of the cytoplasmic (mitochondrial) DNA*

Detailed studies of various physical properties of the cytoplasmic DNA of *L. pictus* are in progress and will be reported elsewhere. Here some preliminary information is given concerning its density, melting behavior, sedimentation properties and microscopic appearance.

(1) *Buoyant density.* In the preparative buoyant density centrifugations in CsCl solution of whole egg homogenates, with radioactive marker DNA included, the $O.D._{260}$ readings consistently show a peak at a higher density (*ca.* 1.70 gm./cm.^{3}) than the peak of radioactivity of the marker (*ca.* 1.69 gm./cm.^{3}). This is illustrated in Figures 2a, 2b. This is also true for the DNA obtained from isolated mitochondria and yolk as shown in Figures 4a, 4b. In further buoyant density centrifugations of purified whole egg DNA of *L. pictus* in the analytical (Beckman Spinco Model E) centrifuge three bands were observed in scans at 265 mμ. The buoyant densities were 1.693, 1.703 and 1.719 gm./cm.^{3} (see Fig. 5). The relative amounts of DNA in these three bands were of the order of 1:7:1. Scans at 280 mμ again revealed three bands in which the ratios of the areas were approximately the same as at 265 mμ. In the same rotor sperm DNA and gastrula DNA form single bands at 1.693 gm./cm.^{3} The 1.703 band evidently represents the bulk of the cytoplasmic DNA. The nature of the 1.719 band is not, as yet, known.

(2) *Melting temperature.* Determinations of melting profiles were made on purified (as described in section A3) DNA preparations from spermatozoa, late gastrulae and unfertilized eggs of *L. pictus*. Sedimentation analysis showed that the latter preparation contained a negligible fraction of intact, *i.e.*, covalently closed, circular DNA. After dialysis and storage in one-tenth strength standard saline citrate (SSC = 0.15 *M* NaCl; 0.015 Na citrate; pH 7.0), the measurements were made in SSC. From the profiles (see Figure 6) the average melting temperatures (Tm) are 84.0° C. for sperm and gastrula DNA and 86.8° C. for the unfertilized egg DNA. In the latter case the value is not entirely attributable to the cytoplasmic DNA since there is some 10% each of nuclear DNA and an unidentified component of a buoyant density in CsCl of 1.719. This may explain the atypical shape of the melting curve of the unfertilized egg DNA (*cf.* also Vinograd and Lebowitz, 1966). From the Tm for the whole egg DNA a guanine-

cytosine (G-C) content of 42% is calculated (Marmur and Doty, 1962; Schildkraut and Lifson, 1965), which approximates the G-C content of 44% calculated (Schildkraut *et al.*, 1962) from the CsCl buoyant density of 1.703 gm./cm.^{3} of the major peak (presumably mitochondrial DNA) obtained from unfertilized eggs. For sperm DNA and gastrula DNA the Tm and the buoyant density indicate a G-C content of 35 and 34%, respectively.

(3) *Sedimentation behavior.* In four separate experiments in which preparations (see section A3) of DNA from whole eggs of *L. pictus* were centrifuged on preformed linear CsCl gradients (1.30 to 1.40 gm./cm.^{3} in the SW-65 rotor for

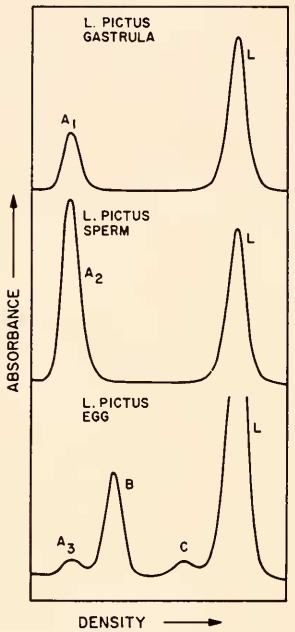

FIGURE 5. Tracings of direct scans at 265 mμ of buoyant density bands (in CsCl) of three preparations of DNA of *L. pictus* after 25 hours of centrifugation in the same rotor at 44,770 rpm in the Beckman model E centrifuge. L: density marker DNA (1.731 gm./cm.^{3}) of *Micrococcus lysodeikticus*. A_1, A_2 and A_3: DNAs identified as nuclear in the three preparations and with similar buoyant densities of 1.693. B: DNA identified as derived from mitochondria and yolk and with a buoyant density of 1.703. C: Unidentified nucleic acid band of buoyant density 1.719.

2 hours at 50,000 rpm, 20° C.) the nuclear DNA sedimented to the bottom of the tube and the cytoplasmic DNA sedimented in two distinct bands, corresponding to sedimentation coefficients of about 23S and 28S, respectively. Approximately equal amounts of the two fractions were obtained, the amounts varying in different preparations. As noted in the introduction, the presence of two such components is indicative of the two forms of circular DNA (twisted and open circles) described by Vinograd *et al.* (1965) and found in mitochondria by van Bruggen *et al.* (1966). Further studies on this material will be reported in a separate paper.

(4) *Electron microscopic observations.* Purified DNAs from whole eggs, from mitochondrial fractions and from gastrulae of *L. pictus* were prepared for electron microscopy according to the method of Kleinschmidt *et al.* (1965). For this purpose a small amount (0.2 ml.) of a solution of ammonium acetate (1.5 *M*; pH 7) containing DNA at about 4 micrograms per ml. and cytochrome *c* at 0.1 mg. per ml. was allowed to flow down an inclined glass slide onto a solution of 0.1 *M* ammonium acetate in a large dish. Electron microscope grids coated with formvar were touched to the surface of the solution, passed through 95% ethanol, 0.0001 *M* uranyl acetate solution in 0.001 *M* HCl and isopentane. The preparations were examined in a Philips EM200 and micrographs taken at a film magni-

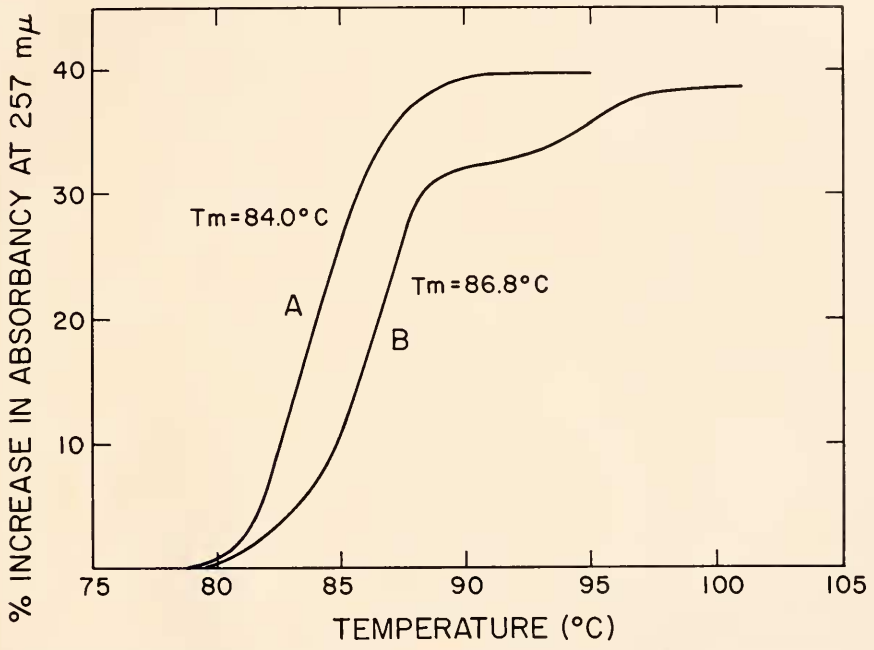

FIGURE 6. Meeting profiles of DNAs (in 0.15 *M* NaCl–0.015 *M* sodium citrate, pH 7) from *L. pictus*. Curve A: DNA from sperm and from gastrulae. Curve B: DNA from whole unfertilized eggs.

fication of 5000 ×. (We are indebted to Mr. James Wetmur of the Division of Chemistry for the use of uranyl acetate in this procedure.)

In the preparations from both whole eggs and mitochondrial fractions the DNA was seen to be present mostly in the form of closed circular filaments, whereas none of these were seen in the preparations from gastrulae. The latter is estimated to contain less than 1% of cytoplasmic DNA. Examples of the circular DNA are shown in Figure 7. Both twisted and open circles are seen. A considerable uniformity of size of circles was observed. Measurements of 144 perimeters gave values ranging from 3.75 to 4.83 microns with a mean of 4.45 and a standard deviation of 0.25. We are indebted to Mr. Donald Blair of the Division of Chemistry for providing us with the foregoing quantitative results.

Discussion

(A) *DNA content*

The present determinations of DNA content of the unfertilized eggs have given values in the general range of those obtained by the more recent workers on sea urchin eggs. However, the values are significantly lower than any previously reported except for that of Marshak and Marshak (1953). By an isotope dilution method they obtained a value of $10 \times$ H (haploid) for *Arbacia punctulata* but attributed most of this to contamination with somatic cells and polar bodies and concluded there was some $3 \times$ to $4 \times$ H of cytoplasmic DNA. They also concluded, from the failure to obtain a Feulgen reaction, that the nucleus of the unfertilized egg lacked DNA. However, measurements by Hinegardner (1961) on isolated nuclei of *E. mathaei* and of *S. purpuratus* showed that these contain $1 \times$ H of DNA and others (*e.g.*, Burgos, 1955) have obtained a positive Feulgen reaction. In the present experiments, the areas under the buoyant density bands in the analytical ultracentrifuge indicate the nuclear DNA to be present in approximately the haploid amount in *L. pictus* eggs.

In the present work evidence has been presented that the material on which the final determinations were made was, in fact, DNA. Also, the monitoring with radioactively labeled DNA permitted an assessment to be made of the effectiveness of recovery during the preparative procedures. This was reinforced by the results of the experiment in which DNA was extracted from eggs in which it had been labeled during oogenesis. It seems reasonable to conclude, then, that the present values of 8.26 pg. and 3.30 pg. per egg for *L. pictus* and *S. purpuratus*, respectively, are close to the actual content of macromolecular DNA in these cells. With regard to other species of echinoids that have been examined, since none of these have an egg size larger than that of *L. pictus* and since the nuclear DNA is closely the same for various species (*cf.* Tyler and Tyler, 1966a), it seems reasonable to expect that the total DNA should be in the same range as the values reported here. The much higher values that have been reported, in the absence of substantial evidence of specificity of the analyses, would seem then to be attributable at least in part to the presence of interfering materials.

In the two species that have been used in the present work the difference in DNA content correlates with differences in egg size. Correlation with egg size may account for the much greater values that have been reported for amphibian eggs in investigations in which attempts have been made to eliminate interfering materials. Thus, Baltus and Brachet (1962) report 0.069 μg. for the axolotl, Haggis (1964) reports $27{,}000 \times$ H for *Rana pipiens*, and Dawid (1965) gives values 600 to $1000 \times$ H for *R. pipiens* and *Xenopus laevis*.

(B) *Presence in mitochondria and yolk*

The present results show, as suggested earlier (Pikó and Tyler, 1965), that the bulk of the cytoplasmic DNA is present in the mitochondria. This accords with the current findings on amphibian (*R. pipiens* and *X. laevis*) eggs by Dawid (1966) who reports that at least two-thirds of the DNA is associated with the mitochondrial particles. As noted in the introduction the general occurrence of DNA in mitochondria is now well established from investigations with various organisms throughout the animal and plant kingdoms.

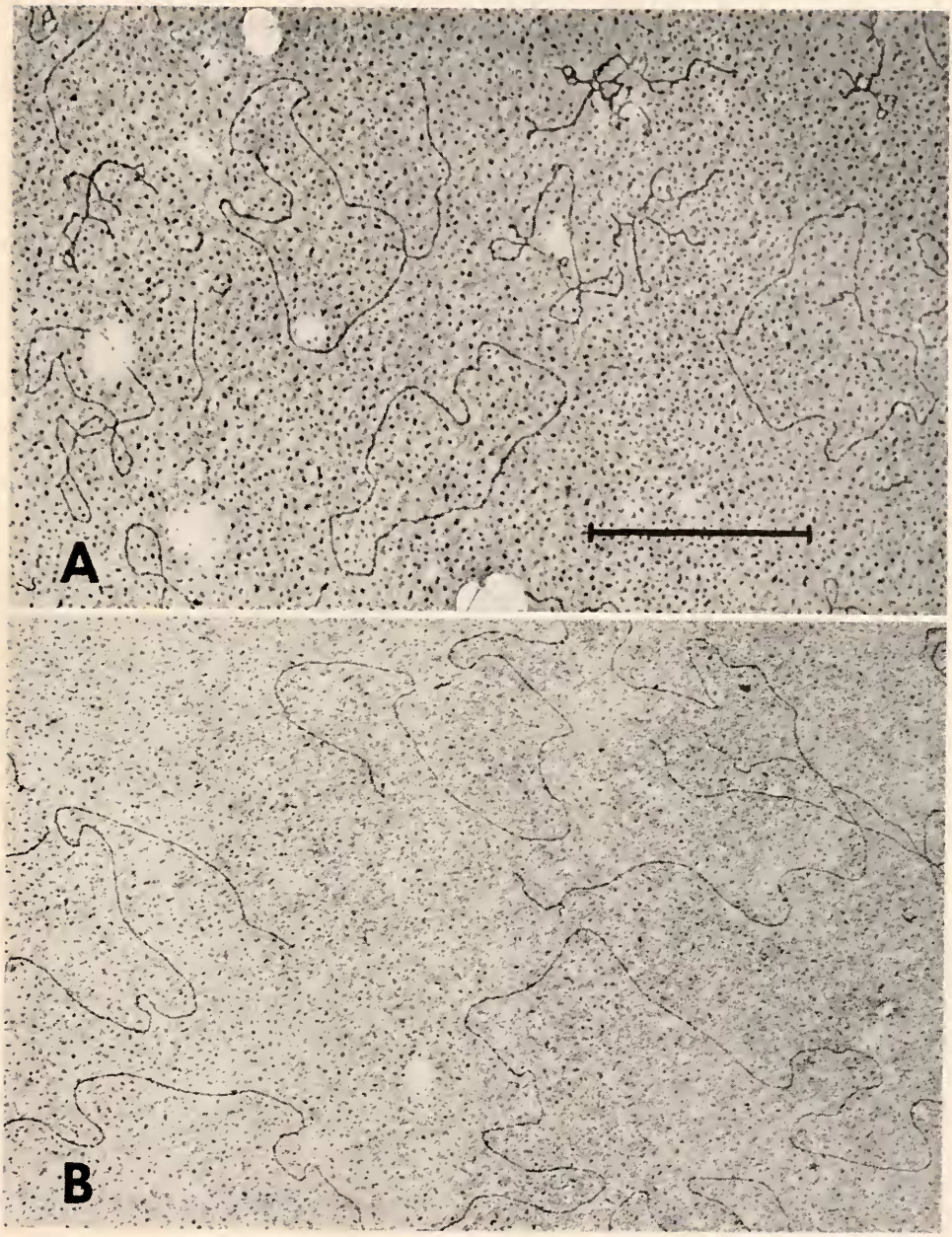

FIGURE 7. Electron micrographs of DNA from mitochondria (A) and of DNA from gastrulae (B) of *L. pictus*. The line represents one micron.

Our results also show the presence of DNA in the preparations of yolk spherules. Although there is a possibility that this DNA is simply adherent to the yolk spherules this seems unlikely in view of the preparative procedures. The evidence accords then with that of Baltus and Brachet (1962), who found about two-thirds of the DNA (as detected by a fluorometric method) of eggs of *Pleurodeles* to be associated with the particles (mostly yolk) sedimentable at low speeds ($280 \times g$). It also accords with the evidence of Brachet and Ficq (1964, 1965), obtained by use of radioactively labeled actinomycin, that DNA is an integral component of the yolk spherules.

There is, in addition, convincing evidence, from studies on frogs (Lanzavecchia, 1960, 1965; Ward, 1962) that at least some of the yolk spherules, or possibly all, are derived from mitochondria. This seems likely to be the case for eggs of animals in general (*cf.* Srivastava, 1965). If one assumes no increase in amount of DNA upon transformation of a mitochondrion into a yolk spherule, then our finding of a much lower content of DNA in the total mass of yolk than in the mass of mitochondria is readily understandable on the basis of the relative volumes of the two particles. Thus in *Lytechinus,* the unfertilized egg contains, by our rough estimate, about 200,000 mitochondria, and 80,000 yolk spherules. From the areas under the bands in the analytical buoyant density centrifugations it appears that seven-ninths of the total DNA, namely 6.4 μg. per 10^6 eggs, is in these particles. This gives 2.3×10^{-17} grams per particle, or 1.4×10^7 daltons.

(C) *Metabolic properties*

The present results showing priming activity for RNA synthesis on the part of the sea urchin cytoplasmic DNA add to the evidence (see introduction) for such activity on the part of the cytoplasmic DNA of eggs of other species (*cf.* Shmerling, 1965 for fish; Baltus *et al.,* 1965 for sea urchins; and Dawid, 1965 for Amphibia). That sea urchin egg mitochondria are capable also of protein synthesis was shown earlier by Nakano and Monroy (1958) and Giudice (1960). It appears, then, that in mitochondria of sea urchin eggs the complete systems of RNA-dependent protein synthesis and DNA-dependent RNA synthesis are present, as has been demonstrated for mitochondria of other origin (*cf.* Kroon, 1963; Kalf, 1964).

Detailed measurements are not as yet available concerning the extent to which the mitochondrial activity accounts for the protein synthesis that occurs upon fertilization in sea urchins, but according to Nakano and Monroy (1958) and Giudice and Monroy (1958) this remains insignificant during the first three or four hours of development. The evidence for potential activity of mitochondrial DNA does not then alter the conclusions that have been drawn earlier from experiments with non-nucleate fragments (Tyler, 1963, 1965; Denny and Tyler, 1964; Brachet, Ficq and Tencer, 1963) and with actinomycin D (Gross and Cousineau, 1963, 1964) of the existence in the unfertilized egg of an inactive ("masked") messenger RNA that becomes active upon fertilization. In fact the experiments with actinomycin D provide particularly strong arguments against the possibility that an activation of mitochondria might be responsible for the great increase in protein synthesis that occurs upon fertilization, inasmuch as it is known (Kalf, 1964) that incorporation of amino acid into protein by intact mitochondria is sensi-

tive to actinomycin. Further arguments are provided by the fact, demonstrated originally by Hultin (1961), that the difference between unfertilized and fertilized eggs is exhibited also by cell-free systems which, from the method of preparation, are evidently free of mitochondria.

(D) *Physical properties*

The buoyant density centrifugation in CsCl solutions in the preparative ultracentrifuge consistently showed the cytoplasmic DNA to have a higher density than the nuclear, in the range of 1.70 to 1.71 gm./cm.^{3} A determination by the analytical ultracentrifuge gives a value of 1.693 for the nuclear and 1.703 for the bulk of the cytoplasmic DNA in *L. pictus* eggs, and a small (10% of the total DNA) band at 1.719. While the latter is a nucleic acid band, as indicated by the $O.D._{260}$–$O.D._{280}$ ratios and other properties, it could be a DNA–RNA hybrid rather than DNA alone. Detailed studies of physical properties of the various components are in progress and will be reported elsewhere.

For the nuclear and cytoplasmic DNA of other species there have been reports both of similarities and of differences in buoyant density. Thus in *Arbacia punctulata*, Carden *et al.* (1965) reported similar buoyant densities for the two DNAs. In *Rana pipiens*, Dawid (1965, 1966) reports that the two DNAs have the same buoyant density (1.702 gm./cm.^{3}) while in *Xenopus laevis* the cytoplasmic DNA is slightly denser (by 0.002 gm./cm.^{3}) than the nuclear. In chickens 1.707 gm./cm.^{3} for the mitochondrial DNA, and 1.698 gm./cm.^{3} for the nuclear, are reported by Rabinowitz *et al.* (1965) and Borst and Ruttenberg (1966). The latter, and also Sinclair and Stevens (1966) report similarity of buoyant density for mitochondrial and nuclear DNAs of various mouse tissues, while Schneider and Kuff (1965) report a somewhat lower buoyant density (1.699 gm./cm.^{3}) for mitochondrial DNA from rat liver as compared with nuclear DNA (1.703 gm./cm.^{3}). In different species of animals, then, the two DNAs may be alike or different in density. The presently available data indicate that even related species may differ in this regard. Even within the same species differences may occur in the buoyant density of mitochondrial DNA, as Mounolou *et al.* (1966) have shown for "petite" mutants of yeast.

Our determinations of melting temperature showed the cytoplasmic DNA to have a Tm of 86.8° C. as compared with 84.0° C. for the nuclear DNA. As noted in the results, this corresponds to a guanine-cytosine content of 42% as compared with 35% for the nuclear DNA. This is in good agreement with the values (44 and 34%, respectively) calculated from the buoyant densities.

The sedimentation experiments revealed two main components in the preparations of cytoplasmic DNA. As noted in the introduction, according to the analysis of Vinograd *et al.* (1965), this, along with other properties, indicated that the sea urchin cytoplasmic DNA might be in the form of circles which could, also, be of twisted and relaxed forms sedimenting at different rates. Examination by electron microscopy has corroborated the circular form. Further studies of this material are in progress. The data reported here show the circles to be of rather uniform size with perimeter close to 4.45 microns. This is near the values reported for mitochondrial DNA of chick and mouse liver and beef heart by van Bruggen *et al.*

(1966) and of mouse and rat liver (and several other tissues) by Sinclair and Stevens (1966).

On the basis of the present evidence from our material, and that of others, it would appear that all of the cytoplasmic DNA may be in circular form, and that filaments may represent breakage due to preparative procedures. In fact the relaxed circles are considered (*cf.* Vinograd *et al.*, 1965) to result from the occurrence of one or more single-strand scissions in the native material and, as preparative procedures improve, the filaments and extended circles become less frequent, as the twisted circles increase proportionately.

On the basis of the present figures it can be estimated that there are only one or two circles (of 4.45 microns perimeter) per mitochondrion of eggs of *L. pictus*. Whether the circular units are genetically alike in all mitochondria is one of the many interesting questions now open for investigation.

Addendum: While this paper was in press, an article appeared by M. M. K. Nass (1966) who reports that there are 2 to 6 circular DNA molecules per mitochondrion in mouse fibroblasts (L cells).

Summary

1. Values of 8.26 ± 0.30 pg. ($9.5 \times$ haploid amount) for *Lytechinus pictus* and 3.30 ± 0.25 pg. ($4.3 \times$ haploid amount) for *Strongylocentrotus purpuratus* have been obtained for the DNA content per egg of these sea urchins. The methods involved repeated CsCl-buoyant density centrifugations, digestion of interfering polysaccharide, and monitoring of the procedures with added radioactively labeled DNA. The final determinations were made on material characterized by several criteria as highly purified.

2. Mitochondrial (M) and yolk (Y) fractions of differentially centrifuged homogenates of *L. pictus* eggs contain the bulk of the cytoplasmic DNA. It is uncertain to what extent the smaller variable amount (11.5 to 26.5%) found in the supernatant may be derived from breakdown of M- and Y-particles.

3. For distribution between M- and Y-fractions the best present value is considered to be about 3:1. Since yolk spherules are approximately one-third as numerous as mitochondria, the amount of DNA is estimated to be the same per particle, namely, 2.3×10^{-17} grams.

4. Evidence is presented that the cytoplasmic DNA of eggs of *L. pictus* and *S. purpuratus* can serve as primer in a DNA-dependent RNA-synthesizing system with approximately the same activity as nuclear DNA.

5. The cytoplasmic DNA of *L. pictus* eggs shows a buoyant density of 1.703 gm./cm.^{3} ac compared with 1.693 for the nuclear. A third nucleic acid band, equal in amount to the nuclear, has been found at a density of 1.719. The amount of nuclear DNA corresponds to the sperm (haploid) value.

6. Melting temperatures in standard saline-citrate are 84.0° C. for sperm and gastrula DNA and 86.8° C. for whole-egg DNA, indicating a guanine-cytosine content calculated from these values as 35% and 42%, respectively. These are similar to the values (34% and 44%) calculated from the buoyant densities.

7. Electron microscopic observations of DNA prepared from mitochondria of *L. pictus* show almost exclusively circles that measure about 4.45 microns in cir-

cumference. It is estimated that there are one or two such circular filaments of double-stranded DNA per mitochondrion or yolk particle.

8. Centrifugation of egg DNA of *L. pictus* in preformed CsCl gradients has revealed two main components with sedimentation coefficients of *ca.* 23S and 28S, indicative of the two forms of circular DNA.

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